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# Mixed Biofilm Formation by Shiga Toxin–Producing *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium Enhanced Bacterial Resistance to Sanitization due to Extracellular Polymeric Substances†

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## ABSTRACT

Shiga toxin–producing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium are important foodborne pathogens capable of forming single-species biofilms or coexisting in multispecies biofilm communities. Bacterial biofilm cells are usually more resistant to sanitization than their planktonic counterparts, so these foodborne pathogens in biofilms pose a serious food safety concern. We investigated how the coexistence of *E. coli* O157:H7 and *Salmonella* Typhimurium strains would affect bacterial planktonic growth competition and mixed biofilm composition. Furthermore, we also investigated how mixed biofilm formation would affect bacterial resistance to common sanitizers. *Salmonella* Typhimurium strains were able to outcompete *E. coli* strains in the planktonic growth phase; however, mixed biofilm development was highly dependent upon companion strain properties in terms of the expression of bacterial extracellular polymeric substances (EPS), including curli fimbriae and exopolysaccharide cellulose. The EPS-producing strains with higher biofilm-forming abilities were able to establish themselves in mixed biofilms more efficiently. In comparison to single-strain biofilms, *Salmonella* or *E. coli* strains with negative EPS expression obtained significantly enhanced resistance to sanitization by forming mixed biofilms with an EPS-producing companion strain of the other species. These observations indicate that the bacterial EPS components not only enhance the sanitizer resistance of the EPS-producing strains but also render protections to their companion strains, regardless of species, in mixed biofilms. Our study highlights the potential risk of cross-contamination by multispecies biofilms in food safety and the need for increased attention to proper sanitization practices in food processing facilities.

Shiga toxin–producing *Escherichia coli* strains are important foodborne pathogens which pose a serious public health concern with a significant financial burden. O157:H7 is the most-commonly identified Shiga toxin–producing *E. coli* serotype associated with foodborne outbreaks and clinical diseases. It was estimated that *E. coli* O157:H7 was responsible for over 73,000 illnesses each year in the United States *(6)*. The symptoms caused by *E. coli* O157:H7 infections range from bloody diarrhea to other, more-severe diseases, such as hemolytic uremic syndrome, a life-threatening complication which is the major cause of kidney failure for children younger than 5 years. Meanwhile, *Salmonella* infections have been reported as the second leading cause of bacterial foodborne illness in the United States, responsible for approximately 11% of all infections caused by foodborne pathogens *(30)*. Over 95% of human salmonellosis cases have been associated with the consumption of contaminated foods, which have included red meat and poultry. Among the many *Salmonella* serotypes, *Salmonella enterica* serovar Typhimurium has been reported as the major and frequent cause of human gastroenteritis. It has been estimated that human infections by *S. enterica* were responsible for approximately 1.4 million clinical cases each year in the United States, resulting in over 17,000 hospitalizations and a financial burden of approximately $2.3 to $3.6 billion on the United States' economy *(10)*. These serious public health concerns and financial consequences highlight the critical need to prevent food contamination by these foodborne pathogens.

Biofilm formation is one of the major strategies that support bacterial survival under adverse circumstances. In the food industry, the attachment of foodborne pathogens on food products and contact surfaces can be enhanced by biofilm formation. Bacterial cells in biofilms are usually more resistant to sanitizing agents than planktonic cells of the same species, and the strong attachment of the biofilm cells on food surfaces also may affect the efficiency of

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TABLE 1. *Bacterial strains, curli and cellulose expression, and measurements of single-species biofilm formation on 96-well polystyrene plates*

| Species | Strain | Expression of: | | Mean no. of CFU/well ± SD ($\times 10^7$) in biofilms[c] | Reference or source |
|---|---|---|---|---|---|
| | | Curli[a] | Cellulose[b] | | |
| *E. coli* O157:H7 | FSIS11 | − | − | 1.58 ± 0.2 | M.H.[d] |
| | FSIS62 | $+_w$ | − | 3.43 ± 1.9 | M.H. |
| *Salmonella* Typhimurium | ST12531 | − | − | 1.16 ± 0.1 | *14* |
| | ST21843 | ++ | + | 5.88 ± 0.7 | *14* |

[a] Curli expression was confirmed by streaking each strain onto Congo red indicator agar plates, and the expression was evaluated based on colony morphology and pigmentation levels on the plates. −, negative expression; $+_w$, weak positive expression; +, positive expression; ++, strong positive expression.

[b] Cellulose expression was confirmed on LB agar plates containing calcofluor dye, and the positive expression of cellulose fimbriae was identified based on the unique morphology of colonies displaying bright fluorescence under UV light.

[c] Each strain was allowed to form single-strain biofilms on 96-well polystyrene plates at room temperature (22 to 25°C) for 72 h. Standard deviation of the mean was calculated from analysis of variance; $n = 6$.

antimicrobial interventions applied to food products for reducing contamination. Thus, biofilm formation by foodborne pathogens is a serious food safety concern, since the detached biofilm cells may become a source of cross-contamination in the food processing environment. The removal and inactivation of foodborne pathogen biofilms are critical for improving hygiene, controlling contamination, and enhancing food safety.

In nature, bacteria are able to form single-species biofilms or, more frequently, coexist in multispecies communities and form mixed biofilms on a wide variety of solid surfaces or food products. It has been shown that multiple bacterial species, including *E. coli* and species of *Salmonella, Staphylococcus, Bacillus, Pseudomonas,* etc., could coexist and form biofilms in meat processing plants *(21)*. More importantly, numerous investigations have shown the presence of *E. coli* O157:H7 and *Salmonella* occurring simultaneously on high percentages of cattle hide, lamb fleece, and carcass samples in multiple commercial meat processing plants of different sizes throughout the United States. These findings indicate that the coexistence of these two important and frequently isolated foodborne pathogens at different meat processing stages poses potential food safety concerns *(2, 3, 5, 14, 16, 25)*.

The available studies further indicate that the coexistence of multiple bacterial species could significantly affect mixed biofilm development and structure. For example, studies focusing on *E. coli* O157:H7 have shown that the surface attachment of an *E. coli* O157:H7 strain unable to form a single-strain biofilm was enhanced by forming mixed biofilms with an *E. coli* O–:H4 companion strain, and bacterial coexistence also enhanced *E. coli* O157:H7 cell survival in mixed biofilms when treated with 5% hydrogen peroxide *(34)*. Similarly, preformed *Acinetobacter calcoaceticus* biofilms have been shown to enhance *E. coli* O157:H7 colonization on solid surfaces under both static and dynamic growth conditions *(12)*. Moreover, *Salmonella* Typhimurium was found to be able to outcompete *E. coli* in heterologous infections and displace *E. coli* cells when the *Salmonella* strain formed biofilms on HEp-2 cells *(9)*. However, an area not well explored is the effect of coexistence of *E. coli* O157:H7 and *Salmonella* Typhimurium, which has been observed in multiple commercial meat processing plants, on bacterial planktonic growth competition and dual-species biofilm formation on solid surfaces. Nor is it understood how mixed biofilm formation would affect the resistance of these foodborne pathogens to common sanitizers. The interspecies relationships within the multispecies community could profoundly affect biofilm development and its composition, as well as pathogen sensitivity to sanitization. Research in this area should be of great interest from a food safety standpoint, because the coexistence of multiple bacterial species is frequently observed in commercial food processing plants. Therefore, the objective of this study was to investigate how the interactions between *E. coli* O157:H7 and *Salmonella* Typhimurium strains would affect bacterial growth competition and colonization on solid surfaces, as well as the multispecies biofilm composition. The effects of mixed biofilm formation on pathogen resistance to common sanitizers were also investigated.

## MATERIALS AND METHODS

**Bacterial strains, culture conditions, and curli and cellulose expression.** Two strains each of *E. coli* O157:H7 and *Salmonella* Typhimurium were used for dual-species biofilm formation in the present study (Table 1). The *E. coli* O157:H7 strains were characterized previously *(4, 24, 36),* and their biofilm-forming abilities were evaluated in our recent study *(35)*. Both *Salmonella* Typhimurium strains used in this study were isolated from hide and carcass swabs of beef cattle during harvest at the processing plants *(14)*. The *Salmonella* strains were serotyped with traditional slide agglutination (O-typing) and tube agglutination (flagellar H-typing) methods, using commercial *Salmonella* antisera (Denka Seiken UK Ltd., Coventry, UK) and following the manufacturer's guidelines. For biofilm assays and sanitization studies, bacterial broth cultures at stationary phase were prepared in Lennox broth (LB; Acumedia Manufacturers, Baltimore, MD) without salt (LB-NS), as described previously *(35),* and then further diluted in fresh sterile LB-NS medium for each experiment.

The levels of expression by the selected strains of two bacterial extracellular polymeric substances (EPS) that have been well associated with bacterial biofilm-forming abilities, curli and cellulose fimbriae, were tested. Bacterial curli expression by each

strain was screened as previously described *(13)* using Congo red indicator (CRI) plates composed of 10 g of Casamino Acids (BD, Franklin Lakes, NJ) per liter, 1 g of yeast extract (Acumedia) per liter, 20 g of Bacto agar (BD) per liter, 40 mg of Congo red (Sigma, St. Louis, MO) per liter, and 20 mg of Coomassie brilliant blue (Sigma) per liter. Bacterial cellulose production was determined as previously described *(33)* on LB agar plates containing 200 mg of calcofluor dye (fluorescent brightener 28, Sigma) per liter.

To compare the growth capability of the selected strains while they replicated individually, each strain was grown statically in LB-NS medium at 22 to 25°C, the same experimental condition that would be used in the biofilm assays. Bacterial growth was examined after 24, 48, and 72 h by diluting and plating appropriate dilutions of each individual culture onto tryptic soy agar (Difco, BD, Sparks, MD) plates or MacConkey agar plates (BD). Bacterial growth was enumerated by CFU counts on the agar plates after incubation at 37°C overnight (16 to 18 h).

**Sanitizers.** Vanquish (Total Solutions, Milwaukee, WI) is a quaternary ammonium chloride (QAC)–based commercial sanitizer authorized by the U.S. Department of Agriculture as category D2 for use in meat, poultry, and other food processing plants. Its active ingredients include a mixture of alkylbenzyldimethylammonium chlorides with various even-numbered alkyl chain lengths, which can be used for food contact surfaces without requiring a rinse. A 1:171 dilution of Vanquish in sterile distilled water, which contains 300 parts per million (ppm) active ingredients as recommended by the manufacture, was used for all QAC treatments throughout the study. The chlorine solution at the final concentration of 100 ppm was prepared in sterile distilled water from commercial germicidal bleach (Clorox) as previously described *(35)*. The chlorine level in the solution was confirmed using High Range Free Chlorine Test Strips (LaMotte Co., Chestertown, MD).

**Dual-species biofilm formation by *E. coli* O157:H7 and *Salmonella* Typhimurium strains.** To investigate the effects of interactions between *E. coli* O157:H7 and *Salmonella* Typhimurium strains on bacterial growth competition and mixed biofilm formation, quantitative biofilm assays were performed on 96-well polystyrene plates as previously described *(35)*. Briefly, each bacterial strain was grown overnight (16 to 18 h) at 37°C in LB-NS broth and then diluted in sterile fresh medium. Equal numbers of bacteria ($\approx 5 \times 10^6$ CFU/ml) from one *E. coli* O157:H7 strain and one *Salmonella* Typhimurium strain were mixed at a 1:1 ratio to make dual-strain cultures and added to 96-well flat-bottom polystyrene plates (Costar, Corning, NY) at 200 μl per well. The cultures were grown statically on the plates at 22 to 25°C for 72 h. Samples containing each individual strain or sterile LB-NS broth only were included as study controls. At the end of the incubation period, a 50-μl aliquot of supernatant was gently harvested from each well by aspiration without disturbing cells on the bottom of the well. Supernatants harvested from replicate samples were combined in order to determine bacterial planktonic growth competition. The remaining supernatants in the wells were gently removed by aspiration, and the plates were washed with 200 μl of sterile phosphate-buffered saline (1× PBS, pH 7.2) per well to remove any loosely attached cells. The plates were air dried for 5 min at 22 to 25°C, and then the wells were filled with 200 μl of sterile LB-NS broth per well. The remaining biofilm cells in each well were harvested by scraping the surface of the well with sterile pipette tips and rinsing the well with the LB-NS broth. Bacterial cells harvested from replicated wells were combined and vigorously vortexed to disrupt cell aggregates. The supernatant and biofilm samples were then serially diluted in fresh LB-NS broth and plated onto MacConkey agar plates for colony enumeration after overnight incubation (16 to 18 h) at 37°C. The two bacterial species were distinguished by colony morphology on the plates, as *E. coli* O157:H7 and *Salmonella* Typhimurium strains form red and yellow colonies, respectively. CFU counts and their corresponding dilution factors, as well as colony morphology and pigmentation on the plates, were used to measure bacterial planktonic growth and biofilm development by cell numbers and to determine the species ratio in each fraction harvested from the dual-strain samples. The experiments were repeated three times using independent cultures, and samples were tested in duplicate in each experiment.

**Biofilm formation on solid surfaces with a biofilm preformed by a different species.** To further explore how bacteria would establish themselves and form biofilms on a solid surface with the presence of a biofilm preformed by other species, pure cultures of *E. coli* O157:H7 or *Salmonella* Typhimurium strains were allowed to form single-species biofilms as described above on 96-well plates for 72 h. After the removal of supernatants and loosely attached cells with sterile PBS washing, overnight cultures of the other species were diluted and added to the plates to allow mixed planktonic growth and biofilm formation for another 72 h in the presence of the preexisting biofilms. By the end of the incubation period, bacterial supernatant and biofilm samples were harvested and processed as described above.

**Bacterial survival in single-strain or dual-strain biofilms treated with sanitizers.** The two types of commercial sanitizers described above were applied to test the survival capability of *E. coli* O157:H7 or *Salmonella* Typhimurium cells in single-species or dual-species biofilms. The single-strain or dual-strain biofilms were developed on 96-well polystyrene plates for 72 h as described above. Bacterial supernatants were gently removed by aspiration at the end of the incubation period, and the plates were washed with 200 μl of sterile PBS per well to remove the residual planktonic cells or any loosely attached cells. The plates were air dried for 5 min at 22 to 25°C and then filled with 200 μl of sterile PBS, the 300-ppm QAC solution, or the 100-ppm chlorine solution per well. After 1 min of incubation at 22 to 25°C, PBS or the sanitizing reagent was removed by gentle aspiration, and all samples were neutralized with 200 μl per well of Dey-Engley broth (Beckton Dickinson) supplemented with 0.3% soytone and 0.25% sodium chloride. The remaining cells in each well were harvested as described above, and cells collected from replicated wells that received the same sanitization treatment were combined and then vigorously vortexed to disrupt cell aggregates. Samples were serially diluted in fresh neutralizing broth, and appropriate dilutions of each sample were plated onto MacConkey agar plates. The plates were incubated overnight (16 to 18 h) at 37°C, and the amount of bacteria surviving was determined by colony enumeration on the plates and their corresponding dilution factors. The species ratios of surviving bacteria in the dual-strain samples were calculated based on colony morphology and pigmentation on the plates as described above. The experiments were repeated three times using independent cultures, and samples were tested in duplicate in each experiment.

**Statistical analysis.** A 1-way analysis of variance with Bonferroni's posttest or unpaired *t* test was used to calculate 2-tailed *P* values using Graph Pad Prism 5 software. A *P* value of $<0.05$ was considered statistically significant. Error bars represent means $\pm$ standard deviations (SD), which were calculated from the analysis of variance.

TABLE 2. *Bacterial planktonic growth and mixed biofilm formation on 96-well polystyrene plates*

| Strain combination | Amt of cells in mixture[a] | | | |
|---|---|---|---|---|
| | Planktonic suspension ($\times 10^7$ CFU/ml; $n = 6$) | | Biofilm ($\times 10^6$ CFU/well; $n = 6$) | |
| | *Salmonella* | *E. coli* | *Salmonella* | *E. coli* |
| *Salmonella*/*E. coli*, coinoculated | | | | |
| ST12531/FSIS11 | 20.4 ± 4.9 (92.9) | 1.55 ± 0.9 (7.1) | 7.25 ± 0.6 (38.7) | 11.5 ± 2.8 (61.3) |
| ST12531/FSIS62 | 28.2 ± 1.0 (96.7) | 0.95 ± 0.6 (3.3) | 1.83 ± 0.7 (18.5) | 8.08 ± 0.6 (81.5) |
| ST21843/FSIS11 | 10.1 ± 1.1 (96.7) | 0.35 ± 0.2 (3.3) | 11.3 ± 0.9 (66.3) | 5.75 ± 0.6 (33.7) |
| ST21843/FSIS62 | 11.8 ± 2.3 (100) | NE | 18.2 ± 0 (71.9) | 7.08 ± 0.6 (28.1) |
| *Salmonella* preinoculated | | | | |
| ST12531/FSIS11 | 36.9 ± 1.8 (99.5) | 0.20 ± 0.1 (0.5) | 13.7 ± 0.5 (74.2) | 4.75 ± 0.6 (25.8) |
| ST12531/FSIS62 | 34.0 ± 0.6 (99.6) | 0.15 ± 0.1 (0.4) | 15.6 ± 4.6 (66.3) | 7.92 ± 2.2 (33.7) |
| ST21843/FSIS11 | 0.80 ± 0.6 (100) | NE | 93.7 ± 28.8 (98.2) | 1.75 ± 0.4 (1.8) |
| ST21843/FSIS62 | 0.55 ± 0.4 (100) | NE | 47.3 ± 6.3 (95.5) | 2.25 ± 0.1 (4.5) |
| *E. coli* preinoculated | | | | |
| ST12531/FSIS11 | 20.1 ± 6.5 (74.6) | 6.85 ± 0.1 (25.4) | 7.58 ± 1.1 (23.5) | 24.7 ± 4.5 (76.5) |
| ST12531/FSIS62 | 26.2 ± 1.7 (94.8) | 1.45 ± 0.2 (5.2) | 5.00 ± 0.5 (20.7) | 19.2 ± 3.1 (79.3) |
| ST21843/FSIS11 | 2.50 ± 0.4 (78.1) | 0.70 ± 0.1 (21.9) | 26.0 ± 3.3 (59.2) | 17.9 ± 0.6 (40.8) |
| ST21843/FSIS62 | 1.40 ± 0.9 (100) | NE | 21.8 ± 0.6 (58.5) | 15.4 ± 2.5 (41.5) |

[a] Data are expressed as means of bacterial CFU per well ± standard deviation (% of cells in each fraction). Means of bacterial CFU per well within either planktonic or biofilm fraction showed a significant difference ($P < 0.05$) between the two strains of each strain pair. NE, not enumerable at the applied dilution factors with the plating and colony enumeration method on MacConkey agar plates.

## RESULTS

**Bacterial curli and cellulose expression and single-strain biofilm formation on 96-well polystyrene plates.** The levels of expression of exopolysaccharide cellulose and curli fimbriae by each bacterial strain were screened on calcofluor plates and CRI plates, respectively. *Salmonella* Typhimurium strain ST21843 was the only strain that demonstrated strong cellulose expression on calcofluor plates. On the CRI plates, *E. coli* O157:H7 strain FSIS62 and *Salmonella* Typhimurium strain ST21843 exhibited weak and strong positive expressions of curli fimbriae, respectively (Table 1). The other two strains exhibited negative curli expression on the plates.

Single-strain biofilm formation on 96-well polystyrene plates was measured by cell numbers in order to compare the biofilm-forming ability of each individual *E. coli* O157:H7 and *Salmonella* Typhimurium strain as an initial screening test. The data in Table 1 represent the mean total bacterial cells per well ($\times 10^7$ CFU per well) harvested from biofilms formed by each individual strain. After removing residual planktonic cells and loosely attached bacteria with PBS washing, the cell densities in the biofilms formed on 96-well polystyrene plates reached approximately $1.6 \times 10^7$ or $3.4 \times 10^7$ CFU per well for the two *E. coli* O157:H7 strains and $1.2 \times 10^7$ or $5.9 \times 10^7$ CFU per well for the two *Salmonella* Typhimurium strains. Notably, strains with positive EPS expression on calcofluor or CRI plates also exhibited greater biofilm-forming abilities than the strains with negative EPS expression. In addition, we also compared the bacterial planktonic growth capabilities of these selected strains during replication individually by plating 24-, 48-, and 72-h room temperature cultures onto TSA or MacConkey agar plates. No significant difference in bacterial growth as measured by colony enumeration was observed on either TSA or MacConkey agar plates among the four strains (data not shown).

**Effects of bacterial coexistence on planktonic growth competition and mixed biofilm formation.** To investigate the effects of bacterial coexistence on planktonic growth competition and mixed biofilm development, equal numbers of bacteria ($\approx 5 \times 10^6$ CFU) from one *E. coli* O157:H7 strain and one *Salmonella* Typhimurium strain were coinoculated to make dual-strain cultures and grown statically on 96-well polystyrene plates. After incubation at 22 to 25°C for 72 h, significantly higher numbers of *Salmonella* Typhimurium cells than of *E. coli* O157:H7 cells were observed in all coinoculated dual-species planktonic suspensions (Table 2). After *E. coli* O157:H7 strain FSIS62 was coincubated with *Salmonella* Typhimurium strain ST21843 for 72 h, FSIS62 cells were not enumerable in planktonic suspensions. These observations indicated that the *Salmonella* Typhimurium strains, regardless of their EPS expression status, were able to effectively compete with and outgrow *E. coli* O157:H7 strains in coinoculated suspensions even though all strains reached similar cell densities while cultured individually for 72 h.

However, the composition of 72-h dual-species biofilms exhibited a different pattern of results (Table 2). When the curli- and cellulose-producing *Salmonella* Typhimurium strain ST21843 was coinoculated with the *E. coli* O157:H7 strains, the percentages of *Salmonella* Typhimurium cells comprising the 72-h mixed biofilms were significantly higher than those of *E. coli* O157:H7 cells, occupying 66.3 and 71.9% of the cell populations in the dual-strain biofilms when mixed with *E. coli* O157:H7 strains FSIS11 and FSIS62, respectively. However, when the *Salmonella* Typhimurium

strain ST12531 with dual-negative EPS expression was coinoculated with the same *E. coli* strains, both *E. coli* O157:H7 strains were retained in higher numbers than the *Salmonella* Typhimurium strain ST12531in mixed biofilms. Furthermore, for the *E. coli* strains in these two strain pairs, the percentage of cells of the curli-producing strain FSIS62 recovered from the mixed biofilms (81.5%) was higher than that of strain FSIS11, with a negative curli expression (61.3%). These findings indicated that the structure of the mixed biofilms formed by *Salmonella* Typhimurium and *E. coli* O157:H7 strains was highly dependent on the companion strain's properties, especially the expression of the EPS components.

**Biofilm formation by *E. coli* O157:H7 or *Salmonella* Typhimurium strains on solid surfaces with preexisting biofilms of the other species.** We further explored *E. coli* or *Salmonella* biofilm formation on solid surfaces with the presence of a biofilm preformed by the other species, which mimics a likely event in real commercial meat plants. We first tested the development of *E. coli* O157:H7 biofilms on a preexisting *Salmonella* biofilm that had been developed for 72 h. In all planktonic supernatants from the four different dual-strain pairs, the *Salmonella* Typhimurium cells were present in significantly higher numbers than the *E. coli* O157:H7 cells at 72 h following the inoculation of the *E. coli* strains. When growing in the presence of the biofilm preformed by *Salmonella* Typhimurium strain ST12531, each *E. coli* strain occupied less than 1% of the total cell population in planktonic suspensions (Table 2). *E. coli* O157:H7 cells were not enumerable in planktonic suspensions when the two *E. coli* strains were inoculated onto the preexisting biofilm formed by another *Salmonella* Typhimurium strain, ST21843. Similarly, of these four strain pairs, the *Salmonella* Typhimurium cells were also present in significantly higher numbers in all mixed biofilms when the *Salmonella* Typhimurium strains precolonized the solid surfaces for 72 h prior to the inoculation of the *E. coli* O157:H7 strains. Notably, the curli- and cellulose-producing *Salmonella* Typhimurium strain ST21843 occupied over 98 and 95% of the total biofilm cell populations, while strain ST12531, with negative EPS expression, occupied 74.2 and 66.3% of the biofilm cell populations after the inoculation of *E. coli* O157:H7 strain FSIS11 (with negative curli expression) and strain FSIS62 (with positive curli expression), respectively. Interestingly, the *Salmonella* Typhimurium EPS-producing strain ST21843 occupied much higher cell percentages in mixed biofilms than the other *Salmonella* Typhimurium strain, ST12531, which showed dual-negative EPS expression, when competing with the *E. coli* strains. Likewise, the curli-producing *E. coli* O157:H7 strain FSIS62 was also present in higher cell percentages (33.7 or 4.5%) in mixed biofilms than *E. coli* strain FSIS11, with negative curli expression (25.8 or 1.8%), when the *E. coli* strains were inoculated onto the preexisting biofilms formed by the two *Salmonella* Typhimurium strains. These observations indicated that surface EPS components also played a significant role in bacterial competition with preexisting biofilms formed by other species.

In additional experiments, each *Salmonella* Typhimurium strain was inoculated onto *E. coli* O157:H7 biofilms preformed for 72 h. After incubation for an additional 72 h, both *Salmonella* Typhimurium strains successfully outgrew the precolonized *E. coli* O157:H7 strains in planktonic suspensions (Table 2); therefore, the preexisting *E. coli* O157:H7 biofilms did not inhibit cell growth of the *Salmonella* Typhimurium strains. Biofilm measurements indicated that *Salmonella* Typhimurium strain ST12531, with negative EPS expression, occupied approximately 20% of the cell population in mixed biofilms, while the EPS-producing *Salmonella* Typhimurium strain ST21843 maintained a much higher cell percentage (≈60%) in mixed biofilms after competing with the precolonized *E. coli* cells of either strains. This suggested that the EPS-producing *Salmonella* Typhimurium strain with greater biofilm-forming ability was able to establish itself in mixed biofilms more efficiently when competing with the preformed *E. coli* biofilms. Furthermore, the percentages of *E. coli* O157:H7 cells in mixed biofilms under this experimental condition were generally higher than in the dual-strain biofilms formed by the two species inoculated simultaneously or when the *Salmonella* Typhimurium strains precolonized the surfaces. These observations suggested that coexistence and growth competition in planktonic phase helps the *Salmonella* Typhimurium strains to compete more effectively during mixed biofilm development, while early-stage precolonization favors the *E. coli* O157:H7 strains competing with *Salmonella* Typhimurium strains and helps *E. coli* biofilm maintenance when challenged by inoculation of the *Salmonella* Typhimurium cells.

**Bacterial cell survival in sanitizer-treated biofilms.** We first investigated bacterial resistance to sanitization while they formed single-strain biofilms. Biofilms formed by each individual strain were treated with sterile PBS, 300-ppm QAC solution, or 100-ppm chlorine solution for 1 min at 22 to 25°C. After exposure to the sanitizers, the viable cell counts recovered from biofilms were significantly reduced compared with the cell counts from their respective PBS-treated samples, and thus, the bacterial sanitization resistance of each strain was evaluated by the difference in viable cells recovered from PBS- and sanitizer-treated samples. Figure 1 shows the means of cell reductions (log CFU per well) that were obtained by subtracting the viable cells recovered after sanitizer treatment from the viable cell numbers recovered after PBS washing. Even though significant cell reductions in biofilms were achieved by either sanitizer, the effectiveness of sanitization appeared to be highly strain dependent (Fig. 1). The EPS-producing strains of each species demonstrated significantly higher resistance to the sanitizers ($P < 0.05$).

We further investigated the effects of dual-species biofilm formation on bacterial resistance to sanitizations. *E. coli* O157:H7 and *Salmonella* Typhimurium strains were allowed to form single-strain or dual-strain biofilms via simultaneous inoculation on 96-well plates for 72 h and then treated with PBS or the sanitizers (Fig. 2). With the 300-ppm QAC treatment, very similar viable-cell reductions of

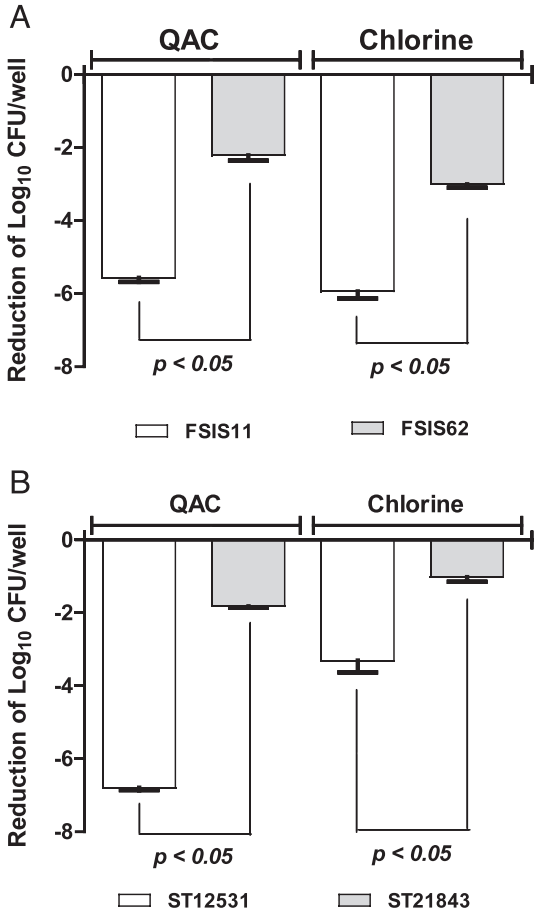


FIGURE 1. *Reductions of bacterial cells in single-strain biofilms formed on 96-well polystyrene plates after treatment with QAC or chlorine solutions. Single-species biofilms of* E. coli *O157:H7 (A) and* Salmonella *Typhimurium (B) strains were treated with sterile PBS or a 300-ppm QAC or 100-ppm chlorine solution. Biofilm cell reductions were calculated by subtracting the numbers of viable cells recovered after sanitizer treatment from the numbers of viable cells recovered after PBS treatment. Data are shown as mean log CFU per well ± SD (*n = 12*). Statistical analysis was performed with the unpaired* t *test to calculate 2-tailed* P *values using Graph Pad Prism 5 software. A* P *value of <0.05 was considered statistically significant.*

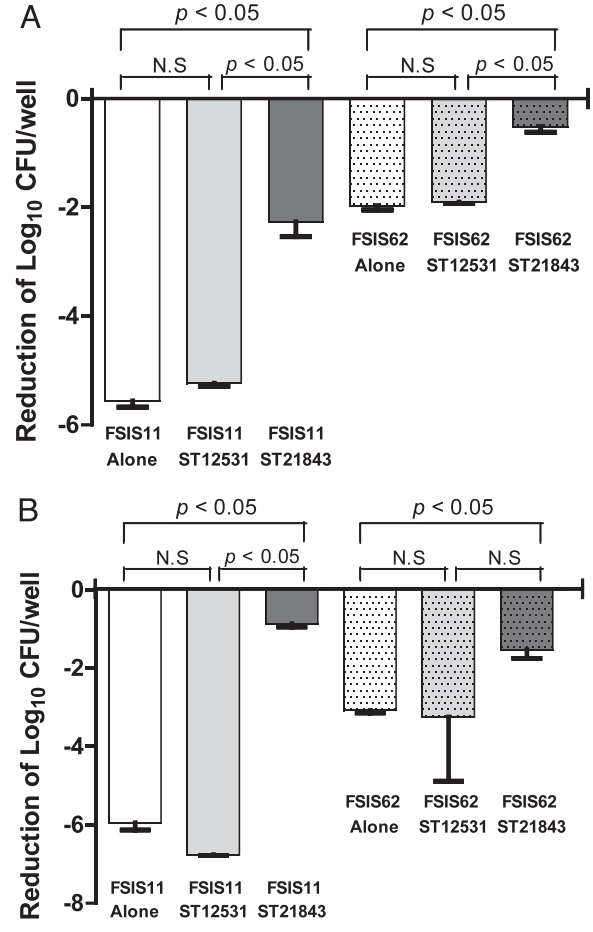


FIGURE 2. *Reduction of* E. coli *O157:H7 cells in single- or dual-strain biofilms formed on 96-well polystyrene plates after treatment with QAC or chlorine solution. Single-species biofilms of* E. coli *O157:H7 strains or mixed biofilms of* E. coli *O157:H7 and* Salmonella *Typhimurium strains were treated with a QAC (A) or chlorine (B) solution that was prepared in sterile distilled water and used at a final concentration of 300 ppm or 100 ppm, respectively. Biofilm cell reductions were calculated by subtracting the number of viable cells recovered after sanitizer treatment from the number of viable cells recovered after PBS treatment. Data are shown as mean log CFU per well ± SD (*n = 12*). Statistical analysis was performed with the unpaired* t *test to calculate 2-tailed* P *values using Graph Pad Prism 5 software. A* P *value of <0.05 was considered statistically significant. N.S, not significant.*

*E. coli* O157:H7 strain FSIS11 were observed for the FSIS11 single-strain biofilm and its mixed biofilms with *Salmonella* Typhimurium strain ST12531 of negative EPS expression. However, significantly less reduction of viable FSIS11 cells ($P < 0.05$) was observed when this *E. coli* O157:H7 strain was present in the mixed biofilm with *Salmonella* Typhimurium EPS-producing strain ST21843. A similar observation was obtained with *E. coli* O157:H7 strain FSIS62. Mixed biofilm formation by *E. coli* strain FSIS62 and *Salmonella* Typhimurium strain ST12531 with negative EPS expression did not affect the FSIS62 cell survival capability compared with that of its single-strain biofilm when treated with the QAC solution, but the resistance of FSIS62 cells to QAC treatment was significantly increased when this *E. coli* strain formed mixed biofilms with *Salmonella* Typhimurium EPS-producing strain ST21843 (Fig. 2A). In addition, significant differences in the QAC resistance of the *E. coli* cells were also observed between mixed biofilms when each *E. coli* strain was paired up with the two different *Salmonella* strains. The QAC resistance of each *E. coli* O157:H7 strain in mixed biofilms together with the EPS-producing *Salmonella* strain ST21843 was significantly higher than that in the mixed biofilm with *Salmonella* strain ST12531 of negative EPS expression.

The resistance of the two *E. coli* strains to the 100-ppm chlorine treatment was also significantly enhanced when these *E. coli* cells were present in mixed biofilms with *Salmonella* Typhimurium EPS-producing strain ST21843, but such increased chlorine resistance was not observed when the two *E. coli* strains formed mixed biofilms with *Salmonella* Typhimurium strain ST12531 of negative EPS expression (Fig. 2B). Companionship with *Salmonella* strain ST21843 also significantly enhanced the chlorine

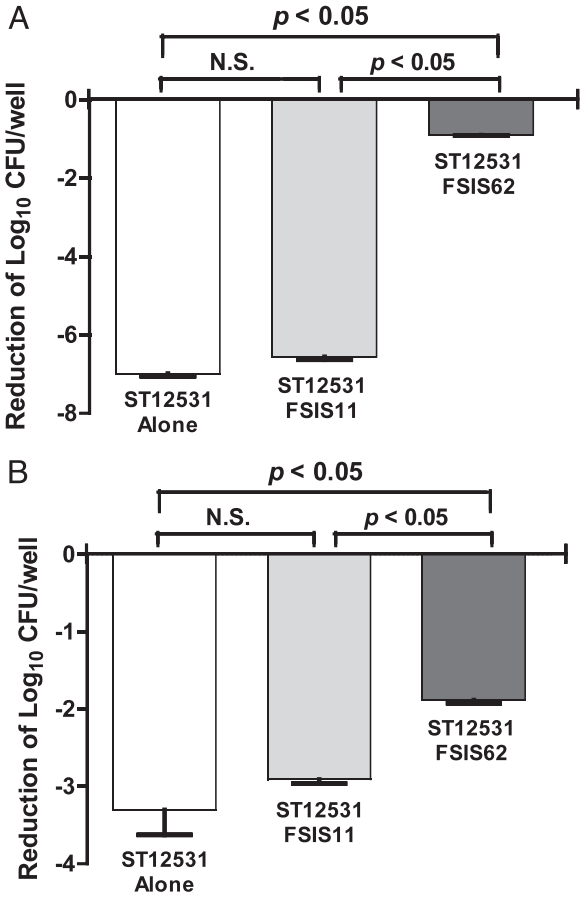


FIGURE 3. *Reduction of* Salmonella *Typhimurium cells in single- or dual-strain biofilms formed on 96-well polystyrene plates after treatment with a QAC or chlorine solution. Single-species biofilms of* Salmonella *Typhimurium strains or mixed biofilms of* Salmonella *Typhimurium and* E. coli *O157:H7 strains were treated with a QAC (A) or chlorine (B) solution that was prepared in sterile distilled water and used at a final concentration of 300 ppm or 100 ppm, respectively. Biofilm cell reductions were calculated by subtracting the number of viable cells recovered after sanitizer treatment from the number of viable cells recovered after PBS treatment. Data are shown as mean log CFU per well ± SD (*n = *12). Statistical analysis was performed with the unpaired* t *test to calculate 2-tailed* P *values using Graph Pad Prism 5 software. A* P *value of <0.05 was considered statistically significant. N.S., not significant.*

resistance of *E. coli* FSIS11 cells compared with that of the FSIS11 cells present in mixed biofilms with *Salmonella* strain ST12531.

The effects of mixed biofilm formation on the sanitizer resistance of the *Salmonella* Typhimurium strains were also investigated. Dual-species biofilm formation with each *E. coli* O157:H7 strain exhibited no effect on the sanitizer resistance of *Salmonella* Typhimurium EPS-producing strain ST21843 compared with the resistance shown by its single-strain biofilm when challenged with either QAC or chlorine solution (data not shown). However, mixed-biofilm formation with the curli-producing *E. coli* strain FSIS62 significantly enhanced the sanitizer resistance of the *Salmonella* Typhimurium strain ST12531 with negative EPS expression. Such increased resistance to QAC and chlorine treatments was clearly observed by comparing the viable-cell reductions in ST12531 single-strain biofilms and in its mixed biofilms with *E. coli* strain FSIS62. In the meantime, dual-species biofilm formation with *E. coli* strain FSIS11, which exhibited negative curli expression, did not increase the survival capability of the ST12531 cells, since the numbers of viable ST12531 cells recovered from its single-strain biofilms or mixed biofilms with strain FSIS11 had no significant difference ($P > 0.05$) when treated with either QAC or chlorine solution. More interestingly, statistical analysis also revealed that the sanitizer resistance of the ST12531 cells was significantly higher in mixed biofilms with *E. coli* strain FSIS62 than in mixed biofilms with *E. coli* strain FSIS11 (Fig. 3).

## DISCUSSION

Biofilms are surface-attached microbial communities that have a tremendous impact on clinical and industrial environments, and therefore, considerable research has been directed at evaluating the impact of foodborne pathogen biofilms on food safety. However, current research efforts have been mostly directed to monospecies biofilms, while apparently, microbial societies in the real environment are heavily biased towards multispecies communities. The results of the many synergistic and antagonistic interactions would determine the dominant species within the mixed biofilms and, consequently, the architecture and activity of the multispecies community. Thus, there is a pressing need for more research efforts directed at understanding the interactions within mixed biofilms and how these interactions could affect the development, composition, and survival of the biofilm community. In the present study, we investigated the competition between *E. coli* O157:H7 and *Salmonella* Typhimurium strains while growing at the planktonic stage and forming mixed biofilms on solid surfaces. In terms of bacterial planktonic growth, our data showed that the *Salmonella* Typhimurium strains were able to effectively outgrow the *E. coli* O157:H7 strains and become the dominant species in the mixture under conditions of simultaneous coinoculation of the two species or of precolonization of the *Salmonella* Typhimurium strains on the solid surfaces. In addition, the *Salmonella* Typhimurium strains could still grow effectively and maintain a higher cell percentage than the *E. coli* O157:H7 strains in the planktonic population even in the presence of a mature *E. coli* biofilm that had been developed for 72 h. The outgrowth of *Salmonella* Typhimurium strains appeared to be a species-related rather than a strain-specific phenomenon, regardless of the EPS expression status.

When the *E. coli* and *Salmonella* cells in dual-species planktonic suspensions were differentiated and quantified by colony enumeration on MacConkey agar plates, in several cases, the *E. coli* O157:H7 cells were not enumerable (Table 2). Such observations did not necessarily imply that *E. coli* O157:H7 cells were all inactivated and not present in planktonic suspensions. The negative enumeration result also could be due to the sensitivity and detection limits of the experimental methods, but these data strongly

implied at the least that *E. coli* O157:H7 cells were present at a much lower level than *Salmonella* cells in the suspensions. On the other hand, with the coinoculated or precolonized *Salmonella* Typhimurium companion strains, the fact that *E. coli* O157:H7 cells were not enumerable (or present at negligible levels) in planktonic suspensions of certain strain pairs but were present in all mixed biofilms suggested that biofilm formation could offer protection and enhance *E. coli* O157:H7 cell survival during competition with *Salmonella* Typhimurium companion strains.

The dual-species biofilm formation as a result of competition between the *E. coli* O157:H7 and *Salmonella* Typhimurium strains was more intriguing, and the composition of the mixed biofilms was apparently associated with bacterial surface EPS expression. Numerous studies have demonstrated the importance of the EPS structures, such as curli fimbriae *(7, 27),* cellulose *(31, 37),* capsular polysaccharide *(8),* and lipopolysaccharide *(1),* in bacterial colonization and biofilm formation. In particular, the contribution of curli fimbriae and exopolysaccharide cellulose to biofilm formation was well appreciated. Our recent study *(35)* also indicated that biofilm formation by *E. coli* O157:H7 and the non-O157 strains on a polystyrene surface was highly strain dependent, and positive curli expression on CRI plates correlated with the greater biofilm-forming ability. Meanwhile, two types of colony morphology have been described in *Salmonella* Typhimurium strains previously *(20).* A typical red, dry, and rough-type colony on CRI agar plates was associated with the coexpression of curli and cellulose fimbriae, while a *Salmonella* Typhimurium smooth and white-type colony was an indication of dual-negative expression of these two EPS structures. In addition, the production of cellulose fimbriae by *Salmonella* Typhimurium strains could also be identified on agar plates containing calcofluor dye, as the cellulose-producing strains would form unique colonies displaying bright fluorescence under UV light *(33).* Furthermore, a positive correlation between colony morphology on indicator agar plates and biofilm formation on solid surfaces was well described by numerous studies *(11, 17, 20, 26).* Therefore, based on colony morphology on the CRI and calcofluor agar plates as a main phenotypic criterion, we selected two *Salmonella* Typhimurium strains that demonstrated either coexpression (strain ST21843) or dual-negative expression (strain ST12531) of curli and cellulose components for the study. The two selected *E. coli* O157:H7 strains exhibited either positive or negative curli expression on CRI plates, but both were identified as strains with negative cellulose expression. Our data for single-strain biofilm formation were consistent with previous findings that the high biofilm-forming ability correlated with the bacterial EPS expression status for both *E. coli* and *Salmonella* strains.

A variety of factors could affect multispecies biofilm formation, including the bacterial species, strain properties, solid surface materials, the sequence of colonization, the age of biofilms, etc. *(9, 12, 21, 32).* Our recent studies analyzing biofilm formation by *E. coli* O157:H7 and *Salmonella* Typhimurium strains on polystyrene plates indicated that these strains developed the highest biofilm mass or reached a plateau of biofilm development after 72 h of incubation at room temperature *(35).* In the present study, we further investigated how the coexistence of *E. coli* O157:H7 and *Salmonella* strains would affect biofilm formation at mature stages (72 h), since bacterial detachment and the resultant contamination often take place after biofilm maturation. Our data indicated that the composition of the 72-h mixed biofilms, unlike that of the mixed-culture supernatants, was highly dependent upon the bacterial EPS properties of the individual companion strains. The EPS-producing strains with higher biofilm-forming abilities were able to establish themselves in mixed biofilms more efficiently. Our further investigation also indicated that, in addition to EPS expression, the sequence of colonization affects mixed biofilm development as well. The bacteria recovered from planktonic suspensions and mixed biofilms were predominantly *Salmonella* Typhimurium cells when the *Salmonella* strains precolonized the solid surfaces (Table 2), suggesting that, in the presence of the preformed *Salmonella* Typhimurium biofilms, the *E. coli* O157:H7 strains were unable to grow efficiently and compete with the precolonized *Salmonella* Typhimurium cells under heterologous culture conditions to replace the preexisting *Salmonella* Typhimurium biofilms. Conversely, early-stage precolonization also enhanced *E. coli* O157:H7 biofilm formation and maintenance during competition with *Salmonella* Typhimurium companion strains. Again, under either experimental condition, bacterial EPS expression appeared to benefit the EPS-producing cells, providing them with higher efficiency to compete with the precolonized cells of the other species.

However, it needs to be acknowledged that the experimental conditions applied in the present study, e.g., solid-surface materials and temperature, are not typical of food processing environments. The current study was designed to investigate bacterial competition at different growth stages and to understand resistance mechanisms that protect bacteria in mixed biofilms against sanitization with biofilm assays performed on 96-well polystyrene plates, which is a common experimental procedure widely used in the biofilm research field. Given the fact that the surface material, temperature, and other environmental factors have significant impacts on biofilm formation, we are currently investigating bacterial competition and mixed biofilm formation at different temperatures on various materials commonly used in the food industry, e.g., polyvinyl chloride, stainless steel, etc.

Many virulent foodborne pathogens, including *E. coli* O157:H7 and *Salmonella* Typhimurium, are able to cause severe human illness with low infectious doses, so bacterial biofilm cells that survive the sanitization procedure present a serious food safety concern through cross-contamination. Previous studies have shown that EPS expression not only increased bacterial biofilm-forming ability but also enhanced biofilm resistance to sanitization. For example, curli production was found to increase the resistance of *E. coli* O157:H7 biofilms to chlorine treatment *(28).* Positive curli expression also improved *E. coli* cell survival in the presence of toxic compounds, such as nickel *(15).* Our recent study *(35)* indicated that the resistance of biofilms formed by *E. coli* O157:H7 and the non-O157 strains to chlorine and QAC treatments was highly strain dependent,

which was also associated with bacterial curli expression. In the present study, our data regarding the sanitizer resistance of *E. coli* and *Salmonella* Typhimurium single-strain biofilms was in agreement with the above-described findings, as the EPS-producing strains exhibited significantly higher resistance to chlorine and QAC treatments than strains of the same species with negative EPS expression (Fig. 1).

In terms of bacterial sanitizer resistance in mixed biofilms, a previous study *(34)* demonstrated that the persistence and hydrogen peroxide resistance of *E. coli* O157:H7 cells in dual-strain biofilms was greatly influenced by the type of the companion strains rather than the total mass of the mixed biofilms. The mechanisms responsible for such enhanced defense capability might also involve the composition and expression of the bacterial surface EPS components, which vary with the bacterial strain and species, the environmental conditions, and the age of the biofilm. Mature biofilms are generally more resistant to physical and chemical stresses due to the strong three-dimensional structure composed of multiple layers of bacterial cells with well-expressed extracellular substances. Therefore, to study the effect of EPS expression on biofilm sanitization resistance, we used mature dual-strain biofilms that had been developed for 72 h to investigate how dual-species biofilm formation and the EPS expression by each companion strain in the mixed biofilms would affect bacterial resistance to the common sanitizers. In addition, for better observation of sanitization resistance and any potentially increased bacterial survival in mixed biofilms, we selected the minimal QAC contact time (1 min) recommended by the manufacturer and, also, a 1-min exposure time for the chlorine solution, which was the middle time point within our biofilm inactivation curve by chlorine treatment (data not shown). Our data showed that both *E. coli* strains obtained significantly enhanced resistance ($P < 0.05$) by forming mixed biofilms with the EPS-producing *Salmonella* Typhimurium strain but not with the *Salmonella* strain of negative EPS expression. Likewise, the *Salmonella* Typhimurium strain with negative EPS expression exhibited significantly enhanced resistance to both sanitizers by forming mixed biofilms with the curli-producing *E. coli* strain but not with the *E. coli* strain of negative curli expression. In addition, the comparison of sanitizer resistance between the mixed biofilms showed that both *E. coli* strains and *Salmonella* strain ST12531 each obtained significantly enhanced survival capability when each of these strains was present in mixed biofilms together with an EPS-producing companion strain of the other species, compared with their survival in mixed biofilms with a partner strain of negative EPS expression. Overall, these observations clearly indicated that bacterial EPS components not only enhance the sanitizer resistance of the EPS-producing strains in biofilms but also render protection against sanitization to their companion strains in mixed biofilms. The mutual protective effects between *E. coli* O157:H7 and *Salmonella* strains indicated that the EPS protection against sanitization was not species restricted.

It is worth noting that biofilms also provide an ideal microenvironment that could lead to horizontal transfer among bacteria of genetic materials of various origins by physical contact. Increased transfer of multidrug resistance plasmids has been demonstrated in *E. coli* and *Staphylococcus aureus* biofilms *(18, 29)*. Furthermore, the available results have shown horizontal transfer of nonconjugative plasmids between *E. coli* cells in colony biofilms *(19)*. Conversely, previous studies more relevant to our research indicated the importance of conjugation as a mechanism that enabled a non–biofilm-forming *E. coli* strain to become a significant component in a mixed biofilm after the acquisition of a plasmid encoding type 3 fimbrium genes, a critical genetic determinant for biofilm formation *(23)*. In addition, a conjugative F plasmid was demonstrated to be an external activator which was involved in curli production and thereby promoted *E. coli* biofilm maturation *(22)*. In the present study, the increased sanitizer resistance of EPS-negative strains could also be a result of horizontal transfer of genetic materials within the microenvironment of mixed biofilms, since many EPS genetic determinants are encoded on plasmids. Further studies are required to investigate the molecular basis for such increased resistance within unique multispecies bacterial communities.

In summary, our results demonstrated that *E. coli* and *Salmonella* EPS components play critical roles in mixed biofilm development, as well as in competition with preexisting biofilms in multispecies communities. The EPS-producing strains with greater biofilm-forming abilities were able to establish themselves in mixed biofilms and compete with preformed biofilms more efficiently. In addition, EPS components not only enhanced the sanitizer resistance of the EPS-producing strains but also protected their companion strains of other species in mixed biofilms. The high potential for biofilm formation by foodborne pathogens and the increased resistance of biofilms to environmental stresses highlights the importance of properly sanitizing food processing equipment and food contact surfaces in order to inactivate biofilm cells. Biofilms in natural environments are most likely composed of multiple species of microorganisms, and the synergistic–antagonistic interactions among these organisms would determine the architecture and activity of the biofilm community. Accordingly, bacterial coexistence, the composition of mixed biofilms, and the effects of interspecies interactions on bacterial sanitizer resistance should be taken into consideration when designing proper sanitization procedures and benchmarking hygienic standards in the food processing environment.

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## REFERENCES

1. Anriany, Y., S. N. Sahu, K. R. Wessels, L. M. McCann, and S. W. Joseph. 2006. Alteration of the rugose phenotype in *waaG* and *ddhC* mutants of *Salmonella enterica* serovar Typhimurium DT104 is associated with inverse production of curli and cellulose. *Appl. Environ. Microbiol.* 72:5002–5012.
2. Arthur, T. M., J. M. Bosilevac, D. M. Brichta-Harhay, N. Kalchayanand, D. A. King, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2008. Source tracking of *Escherichia coli* O157:H7

and *Salmonella* contamination in the lairage environment at commercial U.S. beef processing plants and identification of an effective intervention. *J. Food Prot.* 71:1752–1760.
3. Barkocy-Gallagher, G. A., T. M. Arthur, M. Rivera-Betancourt, X. Nou, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2003. Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J. Food Prot.* 66:1978–1986.
4. Bono, J. L., J. E. Keen, M. L. Clawson, L. M. Durso, M. P. Heaton, and W. W. Laegreid. 2007. Association of *Escherichia coli* O157:H7 *tir* polymorphisms with human infection. *BMC Infect. Dis.* 7:98.
5. Bosilevac, J. M., T. M. Arthur, J. L. Bono, D. M. Brichta-Harhay, N. Kalchayanand, D. A. King, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2009. Prevalence and enumeration of *Escherichia coli* O157:H7 and *Salmonella* in U.S. abattoirs that process fewer than 1000 head of cattle per day. *J. Food Prot.* 72:1272–1278.
6. Clawson, M. L., J. E. Keen, T. P. Smith, L. M. Durso, T. G. McDaneld, R. E. Mandrell, M. A. Davis, and J. L. Bono. 2009. Phylogenetic classification of *Escherichia coli* O157:H7 strains of human and bovine origin using a novel set of nucleotide polymorphisms. *Genome Biol.* 10:R56.
7. Collinson, S. K., S. C. Clouthier, J. L. Doran, P. A. Banser, and W. W. Kay. 1996. *Salmonella* enteritidis *agfBAC* operon encoding thin, aggregative fimbriae. *J. Bacteriol.* 178:662–667.
8. De Rezende, C. E., Y. Anriany, L. E. Carr, S. W. Joseph, and R. M. Weiner. 2005. Capsular polysaccharide surrounds smooth and rugose types of *Salmonella enterica* serovar Typhimurium DT104. *Appl. Environ. Microbiol.* 71:7345–7351.
9. Esteves, C. L., B. D. Jones, and S. Clegg. 2005. Biofilm formation by *Salmonella enterica* Serovar Typhimurium and *Escherichia coli* on epithelial cells following mixed inoculations. *Infect. Immun.* 73:5198–5203.
10. Foley, S. L., and A. M. Lynne. 2008. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *J. Anim. Sci.* 86(14 Suppl.):E173–E187.
11. Gerstel, U., and U. Römling. 2003. The *csgD* promoter, a control unit for biofilm formation in *Salmonella* Typhimurium. *Res. Microbiol.* 154:659–667.
12. Habimana, O., E. Heir, S. Langsrud, A. W. Asli, and T. Møretrø. 2010. Enhanced surface colonization by *Escherichia coli* O157:H7 in biofilms formed by an *Acinetobacter calcoaceticus* isolate from meat-processing environments. *Appl. Environ. Microbiol.* 76:4557–4559.
13. Hammar, M., Z. Bian, and S. Normark. 1996. Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 93:6562–6566.
14. Harhay, D. M., M. N. Guerini, T. M. Arthur, J. M. Bosilevac, N. Kalchayanand, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2008. *Salmonella* and *Escherichia coli* O157:H7 contamination on hides and carcasses of cull cattle presented for slaughter in the United States: an evaluation of prevalence and bacterial loads by immunomagnetic separation and direct plating methods. *Appl. Environ. Microbiol.* 74:6289–6297.
15. Hidalgo, G., X. Chen, A. G. Hay, and L. W. Lion. 2010. Curli produced by *Escherichia coli* PHL628 provide protection from Hg(II). *Appl. Environ. Microbiol.* 76:6939–6941.
16. Kalchayanand, N., T. M. Arthur, J. M. Bosilevac, D. M. Brichta-Harhay, M. N. Guerini, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2007. Microbiological characterization of lamb carcasses at commercial processing plants in the United States. *J. Food Prot.* 70:1811–1819.
17. Kim, S. H., and C. I. Wei. 2007. Biofilm formation by multidrug-resistant *Salmonella enterica* serotype Typhimurium phage type DT104 and other pathogens. *J. Food Prot.* 70:22–29.
18. Król, J. E., H. D. Nguyen, L. M. Rogers, H. Beyenal, S. M. Krone, and E. M. Top. 2011. Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. *Appl. Environ. Microbiol.* 77:5079–5088.
19. Maeda, S., M. Ito, T. Ando, Y. Ishimoto, Y. Fujisawa, H. Takahashi, A. Matsuda, A. Sawamura, and S. Kato. 2006. Horizontal transfer of nonconjugative plasmids in a colony biofilm of *Escherichia coli*. *FEMS Microbiol. Lett.* 255:115–120.
20. Malcova, M., H. Hradecka, R. Karpiskova, and I. Rychlik. 2008. Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: identification of a new colony morphology type and the role of *SGI1* in biofilm formation. *Vet. Microbiol.* 129:360–366.
21. Marouani-Gadri, N., G. Augier, and B. Carpentier. 2009. Characterization of bacterial strains isolated from a beef-processing plant following cleaning and disinfection—influence of isolated strains on biofilm formation by Sakaï and EDL 933 *E. coli* O157:H7. *Int. J. Food Microbiol.* 133:62–67.
22. May, T., and S. Okabe. 2008. *Escherichia coli* harboring a natural IncF conjugative F plasmid develops complex mature biofilms by stimulating synthesis of colanic acid and curli. *J. Bacteriol.* 190:7479–7490.
23. Ong, C. L., S. A. Beatson, A. G. McEwan, and M. A. Schembri. 2009. Conjugative plasmid transfer and adhesion dynamics in an *Escherichia coli* biofilm. *Appl. Environ. Microbiol.* 75:6783–6791.
24. Paton, A. W., and J. C. Paton. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA*, enterohemorrhagic *E. coli hlyA,* $rfb_{O111}$, and $rfb_{O157}$. *J. Clin. Microbiol.* 36:598–602.
25. Rivera-Betancourt, M., S. D. Shackelford, T. M. Arthur, K. E. Westmoreland, G. Bellinger, M. Rossman, J. O. Reagan, and M. Koohmaraie. 2004. Prevalence of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in two geographically distant commercial beef processing plants in the United States. *J. Food Prot.* 67:295–302.
26. Römling, U. 2005. Characterization of the rdar morphotype, a multicellular behaviour in *Enterobacteriaceae*. *Cell. Mol. Life Sci.* 62:1234–1246.
27. Römling, U., Z. Bian, M. Hammar, W. D. Sierralta, and S. Normark. 1998. Curli fibers are highly conserved between *Salmonella* typhimurium and *Escherichia coli* with respect to operon structure and regulation. *J. Bacteriol.* 180:722–731.
28. Ryu, J. H., and L. R. Beuchat. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. *Appl. Environ. Microbiol.* 71:247–254.
29. Savage, V. J., I. Chopra, and A. J. O'Neill. 2013. *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrob. Agents Chemother.* 57:1968–1970.
30. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.
31. Solano, C., B. García, J. Valle, C. Berasain, J. M. Ghigo, C. Gamazo, and I. Lasa. 2002. Genetic analysis of *Salmonella* enteritidis biofilm formation: critical role of cellulose. *Mol. Microbiol.* 43:793–808.
32. Toupoint, N., V. Mohit, I. Linossier, N. Bourgougnon, B. Myrand, F. Olivier, C. Lovejoy, and R. Tremblay. 2012. Effect of biofilm age on settlement of Mytilus edulis. *Biofouling* 28:985–1001.
33. Uhlich, G. A., P. H. Cooke, and E. B. Solomon. 2006. Analyses of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. *Appl. Environ. Microbiol.* 72:2564–2572.
34. Uhlich, G. A., D. P. Rogers, and D. A. Mosier. 2010. *Escherichia coli* serotype O157:H7 retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. *Foodborne Pathog. Dis.* 7:935–943.
35. Wang, R., J. Bono, N. Kalchayanand, S. Shackelford, and D. Harhay. 2012. Biofilm formation by Shiga-toxin producing *Escherichia coli* O157:H7 and non-O157 strains and their tolerance to sanitizers commonly used in food processing environment. *J. Food Prot.* 75: 1418–1428.
36. Westerman, R. B., Y. He, J. E. Keen, E. T. Littledike, and J. Kwang. 1997. Production and characterization of monoclonal antibodies specific for the lipopolysaccharide of *Escherichia coli* O157. *J. Clin. Microbiol.* 35:679–684.
37. Zogaj, X., M. Nimtz, M. Rohde, W. Bokranz, and U. Römling. 2001. The multicellular morphotypes of *Salmonella* typhimurium and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol. Microbiol.* 39:1452–1463.